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Article

Transcriptional Cofactor TBLR1 Controls Lipid Mobilization in White Adipose Tissue

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SUMMARY

Lipid mobilization (lipolysis) in white adipose tissue (WAT) critically controls lipid turnover and adiposity in humans. While the acute regulation of lipolysis has been studied in detail, the transcriptional determinants of WAT lipolytic activity remain still largely unexplored. Here we show that the genetic inactivation of transcriptional cofactor transducin beta-like-related 1(TBLR1) blunts the lipolytic response of white adipocytes through the impairment of cAMP-dependent signal transduction. Indeed, mice lacking TBLR1 in adipocytes are defective in fasting-induced lipid mobilization and, when placed on a high-fat-diet, show aggravated adiposity, glucose intolerance, and insulin resistance. TBLR1 levels are found to increase under lipolytic conditions in WAT of both human patients and mice, correlating with serum free fatty acids (FFAs). As a critical regulator of WAT cAMP signaling and lipid mobilization, proper activity of TBLR1 in adipocytes might thus represent a critical molecular checkpoint for the prevention of metabolic dysfunction in subjects with obesity-related disorders.

INTRODUCTION

White adipose tissue (WAT) has been described as an important endocrine organ controlling systemic lipid metabolism (Galic et al., 2010), and WAT lipid homeostasis is known to represent a critical determinant of body weight, insulin sensitivity, and peripheral energy handling in both mice and humans (Yu and Ginsberg, 2005). Indeed, in humans, a combination of excessive lipid storage and decreased removal leads to obesity and associated comorbidities, including insulin resistance and type 2 diabetes (Langin, 2011), now affecting more than 500 million obese people worldwide (Finucane et al., 2011). Excessive lipid load causes adipocyte stress, which in turn accounts for many adverse effects of obesity, particularly alterations in adipocytokine release and a low-grade inflammatory response, ultimately leading to the development of metabolic dysfunction such as worsened insulin sensitivity and glucose intolerance (Hotamisligil, 2006; Rasouli and Kern, 2008).

Due to their critical role in the maintenance of proper adipocyte function, both storage and release of lipids in WAT are physiologically under tight hormonal control: whereas insulin promotes triglyceride (TG) storage during the postprandial phase, catecholamines trigger the breakdown of TG into glycerol and fatty acids (''lipolysis'') to provide energy substrates for other organs, including liver and skeletal muscle, during fasting (Yu and Ginsberg, 2005).

Upon catecholamine binding to beta-adrenergic receptors, the lipolytic cascade is induced by the activation of adenylate cyclase to trigger cAMP/cAMP-dependent protein kinase A (PKA) signal transduction, ultimately leading to the phosphorylation-dependent activation of critical downstream lipases, most notably adipose TG lipase (ATGL) and hormone-sensitive lipase (HSL), which catalyze the hydrolysis of triacylglycerol and diacylglycerol, respectively (Zechner et al., 2012). Whereas HSL is a direct PKA phosphorylation target, ATGL is indirectly activated through the PKA-mediated phosphorylation of the lipid droplet protein perilipin A and the subsequent release of the perilipin-bound ATGL coactivator comparative gene identification 58 (CGI-58) (Lass et al., 2006).

Although lipolysis in WAT was recently found to critically determine lipid turnover and adiposity in humans (Arner et al., 2011), the molecular determinants of WAT lipolytic activity still remain largely unknown. In this respect, transcriptional cofactor complexes have been identified as important checkpoints in the coordination of metabolic programs in various tissues, including WAT (Herzig et al., 2001; Ostertag et al., 2010; Sommerfeld et al., 2011). Indeed, we have recently identified a role for the transducin beta-like 1(TBL1)/TBL-related 1(TBLR1) transcriptional cofactor complex in liver lipid metabolism (Kulozik et al., 2011), originally identified as a component of the nuclear receptor corepressor (NCoR)/silencing mediator for retinoid and thyroid receptors (SMRT) complex (Guenther et al., 2000). TBL1 levels

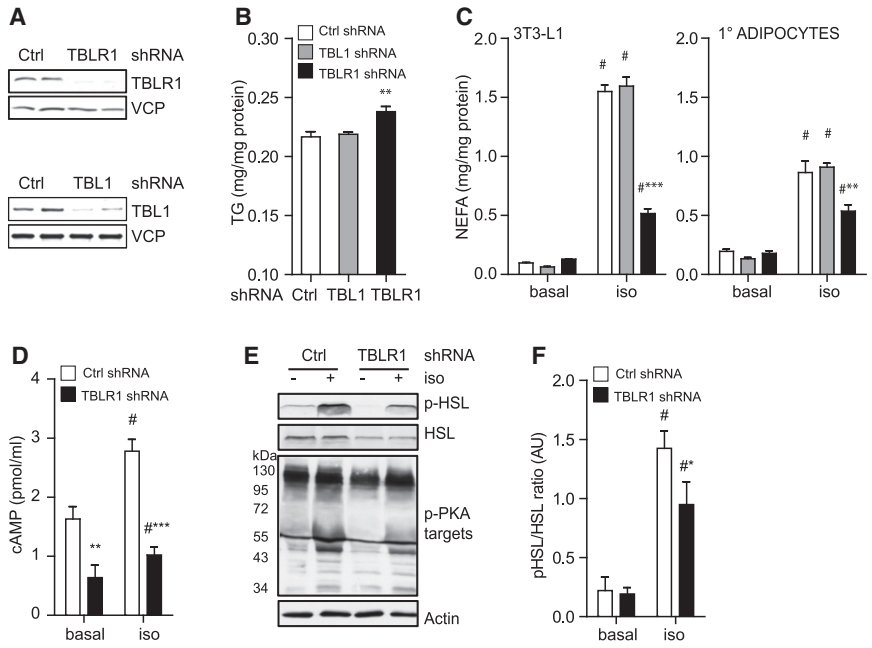

Figure 1. TBLR1 Controls Adipocyte Triglyceride Levels by Regulating Lipolysis

(A) TBL1, TBLR1 and valosin-containing protein (VCP) protein levels in 3T3-L1 adipocytes 3 days after infection with adenoviruses carrying shRNA against TBL1, TBLR1, or a control sequence.

(B) TG levels in the same cells.

(C) Nonesterified fatty acid (NEFA) release from 3T3-L1 and primary adipocytes stimulated with 10 μM isoproterenol for 3 hr (± iso) measured in supernatants of the cells.

(D) cAMP levels of 3T3-L1 adipocytes ± iso.

(E) Immunoblot of phosphorylated PKA targets and HSL (at Serine 660) in 3T3-L1 adipocytes ± iso compared to total HSL and actin levels.

(F) Ratio of S660 phosphorylated HSL to total HSL as measured by quantitative analysis of immunoblots with ImageJ.

All values in bar graphs are expressed as means ± SEM, n = 4, #$p < 0.05$ basal versus stimulated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ control versus TBLR1 shRNA-treated cells. See also Figure S1 and Table S1.

in the liver negatively correlate with hepatic TG content in humans, and TBL1 levels are decreased in liver of mouse models of obesity and steatosis. Accordingly, acute knockdown of TBL1 in mouse livers promotes hepatic steatosis and hypertriglyceridemia, which is—at least in part—explained by reduced fatty acid oxidation due to impaired TBL1 coactivator function for nuclear receptor PPARα. The identification of TBL1/TBLR1 as nuclear receptor-dependent regulators of liver lipid metabolism provided the first indication that these cofactors might fulfill broader functions in the control of energy homeostasis. However, the embryonic lethality of whole-body TBL1 or TBLR1 knockout mice (Perissi et al., 2010) thus far prevented the further investigation of these cofactors in metabolic control.

RESULTS

TBLR1 but Not TBL1 Regulates Lipid Handling in Adipocytes

The recently discovered role of the TBL1/TBLR1 transcriptional complex in the manifestation of hepatic steatosis (Kulozik et al., 2011) initially prompted us to explore potential additional "metaboloregulatory" functions of these cofactors. Given the importance of adipose tissue for systemic energy handling, we thus disrupted the activity of both cofactors by transducing mature 3T3-L1 white adipocytes with adenoviruses expressing TBL1- or TBLR1-specific or nonspecific control short hairpin RNAs (shRNAs). TBL1 and TBLR1 shRNA treatment significantly reduced the protein levels of both cofactors as compared with control shRNA-treated cells (Figure 1A). Neither TBL1 nor TBLR1 knockdown influenced adipogenesis in this system (Figures S1I–S1K available online). Also, knockdown of TBLR1 had no effect on lipogenesis, fatty acid (FA) β-oxidation, glucose uptake, and insulin signaling in the adipocytes (Figure S1A–S1D) but led to a significant elevation of intracellular TG content compared with controls (Figure 1B), indicative of reduced lipid removal from the cells. Indeed, loss of function of TBLR1 significantly blunted beta-adrenoceptor (isoproterenol)-stimulated lipolysis in mature 3T3-L1 white adipocytes (Figures 1C and S1E). The same was true for primary white adipocytes that had been differentiated from the progenitor population within the stromal-vascular fraction of inguinal white fat depots of wild-type mice (Figures 1C and S1E). Importantly, overexpression of TBLR1 in adipocytes caused a reduction in intracellular TG levels and significantly augmented the lipolytic response (Figures S2A and S2B).

Interestingly, deficiency in TBL1 had no effect on either TG content or lipolysis under these conditions (Figures 1B, 1C, and S1E), arguing for a specific regulatory importance of TBLR1 but not TBL1 in white adipocyte lipid metabolism.

TBLR1 Controls Multiple Steps in the Lipolytic Cascade

As stated above, beta-adrenergic receptor activation and subsequent induction of cAMP signaling promotes lipolysis in white adipocytes via the induction of protein kinase A (PKA)-mediated phosphorylation of hormone-sensitive lipase (HSL) (Haemmerle et al., 2002; Osuga et al., 2000) and the lipid droplet protein perilipin, indirectly activating adipocyte triglyceride lipase (ATGL) (Zechner et al., 2009). While inhibition of cellular phosphodiesterase activity had no influence on the TBLR1-mediated inhibition of lipolysis (Figure S1F) and loss of TBLR1 did not alter the PKA holoenzyme subunit composition (Figure S1G), intracellular cAMP levels were found to be lower in mature 3T3-L1 white adipocytes in response to TBLR1 deficiency (Figure 1D). Consistent with these findings, activation of HSL by phosphorylation at Ser660 and total PKA substrate phosphorylation, including perilipin, upon beta-adrenergic stimulation was substantially blunted in 3T3-L1 or primary adipocytes lacking TBLR1 (Figures 1E and 1F and data not shown). In turn, TBLR1 overexpression triggered enhanced HSL as well as total PKA substrate phosphorylation (Figure S2C).

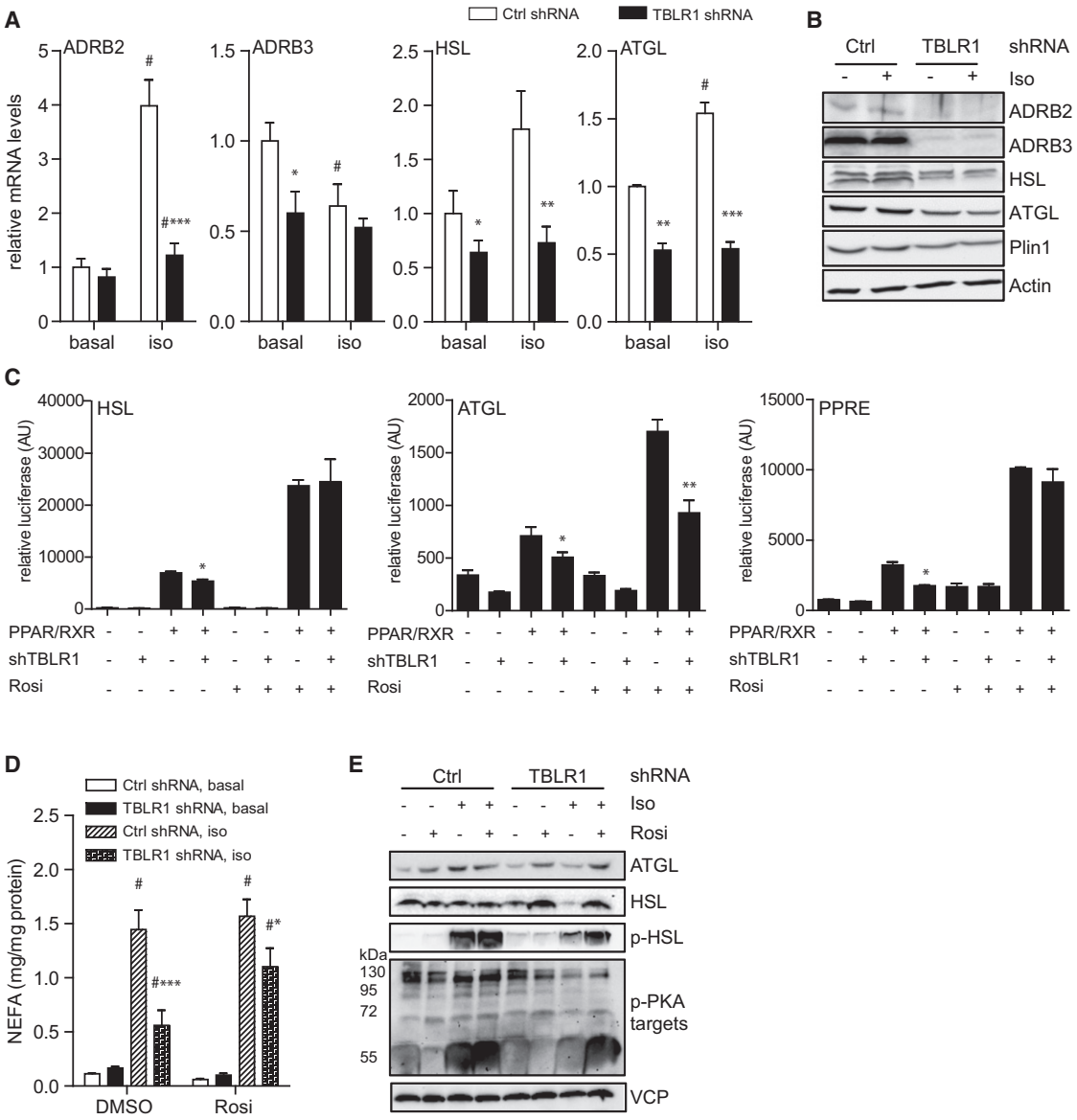

Figure 2. TBLR1 Regulates Lipolytic Gene Expression

(A and B) mRNA and protein expression of beta2- and beta3-adrenergic receptors (ADRB2 and ADRB3), HSL, and ATGL upon TBLR1 knockdown in 3T3-L1 adipocytes ± iso.

(C) HSL, ATGL, and PPRE promoter activity in 3T3-L1 preadipocytes transfected with TBLR1 or control shRNA and PPAR/RXR expression plasmids, treated with or without 1 μM rosiglitazone for 24 hr. Luciferase activity normalized to β-galactosidase and the respective pGL3 empty vector control.

(D) NEFA release from 3T3-L1 adipocytes stimulated with 10 μM isoproterenol for 3 hr in the presence or absence of 1 μM rosiglitazone (rosi) for 24 hr.

(E) Immunoblot of ATGL, HSL, p-HSL, p-PKA target, and VCP levels in the same cells.

All values in bar graphs are expressed as means ± SEM, n = 4, #$p < 0.05$ basal versus stimulated; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ control versus TBLR1 shRNA-treated cells. See also Figure S2.

To further delineate the mechanism of TBLR1 action, we performed global gene expression profiling in TBLR1-deficient and wild-type control adipocytes. Consistent with the significant impact of TBLR1 on adipocyte lipid metabolism, genetic pathway analysis revealed that the pathway cluster containing FA-associated, adipocytokine as well as peroxisome proliferator-activated receptor (PPAR) nuclear receptor pathways was most strongly regulated by TBLR1 deficiency (Table S1 and Figure S1H). As TBLR1 affected lipolysis stimulated by different beta2- and beta3-adrenoceptor-specific agonists as well as by direct adenylate cyclase agonism (Figure S2D), we hypothesized that TBLR1 controls multiple steps in the beta-adrenoceptor signaling cascade. Indeed, TBLR1 deficiency significantly impaired the messenger RNA (mRNA) and protein expression of the beta2- and beta3-adrenergic receptors (ADRB2 and ADRB3) and of HSL and ATGL in this setting (Figures 2A, 2B, and S2J). Consistent with these findings, the activities of HSL and ATGL luciferase promoter constructs were significantly

impaired upon TBLR1-specific shRNA treatment (Figure 2C). In keeping with a role of these genes in the PPARγ nuclear receptor pathway (Anghel et al., 2007; Rodriguez-Cuenca et al., 2012a, 2012b), TBLR1 was found to physically interact with the PPARγ/RXRα complex and to regulate a PPAR response element-dependent reporter gene in mature adipocytes (Figures S2E, S2F, and 2C). Indeed, treatment of mature adipocytes with a PPARγ- but not PPARα- or PPARβ/δ-selective agonist was able to partially rescue the defect in lipolysis and inhibition of target gene expression mediated by TBLR1 deficiency (Figures 2C–2E and S2G–S2I).

Adipocyte-Specific TBLR1 Deficiency Impairs Fasting-Induced Lipid Mobilization

Thus far, our results supported the notion that the cell autonomous transcriptional activity of TBLR1 controls multiple beta-adrenoceptor and postreceptor checkpoints in white adipocytes, thereby coordinating an integrated response of cAMP-dependent free fatty acid (FFA) mobilization. To investigate the functional impact of TBLR1 on WAT energy homeostasis in vivo, we generated adipocyte-specific TBLR1 knockout (ATKO) mice by introducing loxP sites flanking exon 5 of the *Tblr1* gene and crossing homozygous animals with mice carrying Cre recombinase under the control of the aP2 promoter (He et al., 2003), thereby excising exon 5 and creating a stop codon in exon 6 ($Cre^{+}/lox^{+/+}$; Figures S3A and S3B). At 8 weeks of age, ATKO animals had no obvious alterations in body weight, body fat content, and serum parameters (Figure S3C and data not shown). Studies in humans have demonstrated that a chronic lipolytic defect leads to overt adiposity over time (McQuaid et al., 2011). In agreement, 14-week-old ATKO animals showed increased body weight and body fat content, which was also present at thermoneutral animal housing temperatures and developed into a stable body weight difference at older ages (8 months) as compared with their wild-type (WT) littermates (Figure 3A and data not shown). No differences in food intake and fecal energy content were observed in these animal cohorts (Figures S3D and S3E). A 24 hr fasting challenge of 14-week-old animals led to a substantial reduction of abdominal and inguinal fat pad weights in WT mice (Figures 3B–3D), correlating with WAT HSL and PKA substrate phosphorylation (Figure 3F). In contrast, WAT TBLR1 deficiency impaired the fasting-induced fat pad weight reduction, NEFA release, and HSL/PKA substrate phosphorylation (Figures 3B–3F), indicating that TBLR1 is required for efficient lipid mobilization in vivo. Consistent with this assumption, histological examination revealed a significantly increased proportion of large adipocytes in WAT depots from fasted ATKO mice as compared with WT littermates, whereas adipocyte numbers remained unchanged (Figures 3G and S5I). Isoproterenol-injected ATKO mice showed equally impaired cAMP signaling (Figure 3H).

Furthermore, abdominal WAT and brown adipose tissue (BAT) explants from ATKO mice showed a substantially impaired release of NEFA and glycerol in response to beta-adrenoceptor agonist treatment for 3 hr as compared with WT explants (Figures 4A, 4B, S4A, and S4B). Consistent with the in vitro data, loss of TBLR1 in AT depots abolished the isoproterenol-mediated activation of HSL and PKA substrate phosphorylation and led to a reduction in HSL and beta-adrenoceptor expression in ATKO mice (Figures 4C and S4C). Whereas BAT from ATKO mice displayed mildly impaired uncoupling protein 1(UCP1) expression, overall BAT histology and cold-induced increase in oxygen consumption remained unchanged in these animals (Figure S3F, S3G, S5M, and S5N).

Lack of TBLR1 in Adipose Tissue Aggravates Obesity and Metabolic Dysfunction

The importance of defective WAT FA handling for human obesity (Arner et al., 2011; Karpe et al., 2011) next prompted us to explore the contribution of fat-specific TBLR1 activity to obesity-related metabolic dysfunction. To this end, we placed WT and ATKO mice on either low-fat (LDF) or high-fat (HFD) diets (10% or 60% calories from fat, respectively) for 8 weeks. Whereas body weight mildly increased in ATKO animals under LFD conditions as shown above, ATKO mice on a HFD gained significantly more weight than their WT littermates (Figure 5A). The difference in body weight gain was explained by increased adiposity in the ATKO mice as determined by body composition analysis and WAT depot sizes (Figures 5B and S5A–S5D). Also, cell size distribution analysis revealed that ATKO mice had substantially larger abdominal white adipocytes than did control littermates on HFD (Figure 5C and S5F), correlating with reduced lipolysis, HSL, and PKA substrate phosphorylation, and lower expression of beta-adrenoceptors, HSL, and ATGL (Figures 5D, S5E, and S5G). In accordance with the increased adiposity in ATKO mice, both glucose tolerance and systemic insulin sensitivity were significantly impaired upon adipocyte TBLR1 deficiency (Figures 5G–5I and S5H), correlating with elevated serum leptin and resistin levels, ectopic lipid accumulation in muscle, WAT inflammatory marker gene expression, and increased appearance of ''crown-like'' structures as compared to controls, indicative of increased WAT damage (Figures 5E, 5F, and S5J–S5L). These data suggest that the lack of TBLR1 transcriptional activity in WAT abrogates adipocyte lipid mobilization, thereby contributing to excess adiposity and metabolic dysfunction upon exposure to fat-rich diet.

TBLR1 Expression Is Increased under Lipolytic Conditions in Humans

To finally explore the relevance of our findings for the human situation, we analyzed TBLR1 mRNA expression in obese female patients who underwent a controlled weight reduction program (Rudofsky et al., 2011) (Table S2, part A). Consistent with mouse data showing elevated TBLR1 mRNA levels during lipolytic conditions like fasting, beta-adrenoceptor agonist treatment, and lipopolysaccharide (LPS) injection (Figures S6A–S6C), TBLR1 mRNA expression in WAT was significantly increased in patients after weight reduction as compared to the pre-weight-loss stage (Figure 6A), substantiating the notion that lipid mobilization is associated with elevation of TBLR1 expression in human and mouse WAT. Indeed, TBLR1 but not TBL1 mRNA levels were positively correlated with serum FFA and adiponectin levels and negatively correlated with c-reactive protein (CRP) over a wide body mass index (BMI) range (18.4 to 62.7 kg/m^2) in a female patient cohort (Figures 6B–6D and Table S2, part B). Consistent with the correlation of WAT TBLR1 mRNA expression with beta2-adrenoceptor mRNA and FFA levels in mice (Figures S6D and S6E), TBLR1 mRNA expression also significantly

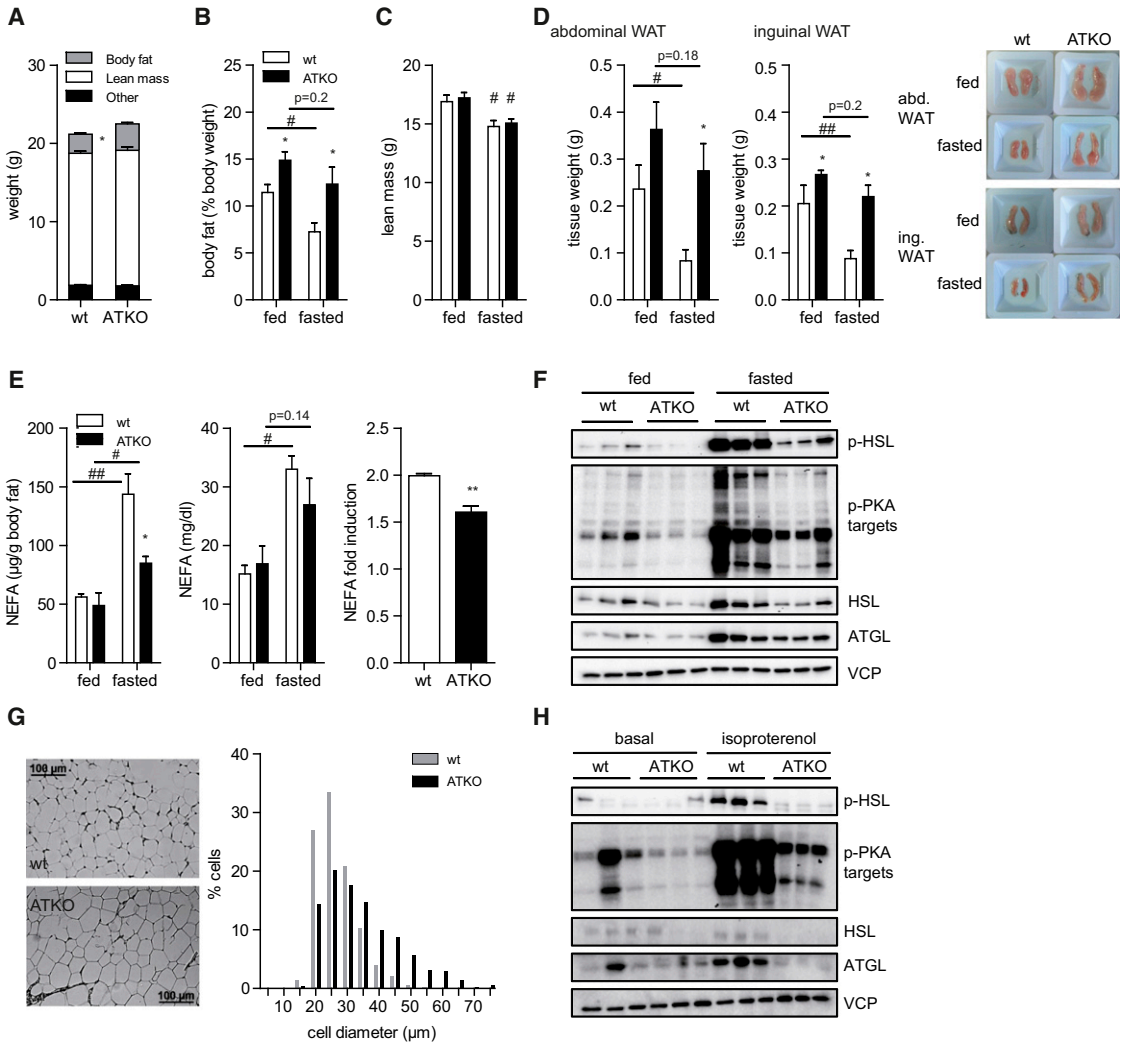

Figure 3. Adipocyte-Specific TBLR1 Knockout Mice Display Increased Body Fat and Adipocyte Size due to Reduced Lipolysis

(A) ECHO-MRI body composition analysis of 14-week-old WT and adipocyte-specific TBLR1 knockout (ATKO) mice.

(B and C) Body fat content (B) and lean mass (C) of 14-week-old ATKO and WT mice before and after 24 hr fasting measured by ECHO-MRI.

(D) Abdominal and inguinal WAT depot weights and macroscopic appearance in the same mice.

(E) Lipolysis before and after 24 hr fasting calculated by total serum NEFA divided by total body fat mass before fasting (first panel), serum NEFA levels (second panel), and relative induction by fasting (third panel).

(F) Immunoblot of p-HSL, p-PKA targets, HSL, ATGL, and VCP in abdominal WAT of the same animals.

(G) H&E-stained abdominal WAT slices and quantitation of adipocyte diameter in 8-week-old 48-hr-fasted mice.

(H) Immunoblot of p-HSL, p-PKA targets, HSL, ATGL, and VCP in abdominal WAT of male mice injected with 10 mg/kg isoproterenol for 30 min.

All values in bar graphs are expressed as means ± SEM, n = 6; #p < 0.05, ##p < 0.01 fed versus fasted, *p < 0.05, **p < 0.01 WT versus ATKO. See also Figure S3.

correlated with both beta1 and beta2 adrenoceptor levels in the same patients (Figures 6E and 6F), further supporting the hypothesis that TBLR1 function in WAT is conserved and coupled to adipose tissue lipid homeostasis and the lipolytic pathway also in humans.

DISCUSSION

Proper metabolic flexibility to adapt to varying conditions of energy availability is a crucial prerequisite for metabolic health. Indeed, aberrant lipid turnover and particularly relative impairment of WAT FFA release represent critical features of both murine and human obesity, triggering WAT inflammation, dysfunctional adipocytokine signaling, and eventually systemic insulin resistance and glucose intolerance (Arner et al., 2011; Samuel and Shulman, 2012).

In the current study, the transcriptional cofactor TBLR1 was identified as a central player in adipocyte lipid metabolism. In mice, adipose tissue TBLR1 deficiency had a substantial impact on some of the most central pathways to adipocyte biology, including fatty acid metabolism and adipocytokine expression (Dahlman and Arner, 2010). In addition to reduced adrenoceptor levels, TBLR1 knockdown particularly led to reduced ATGL and HSL expression in adipocytes, thereby targeting the lipolytic

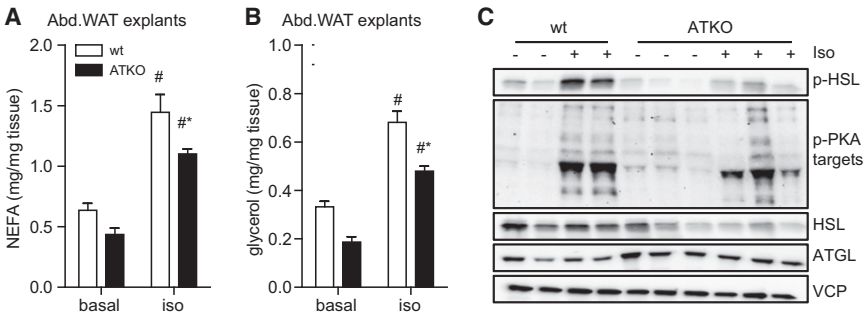

Figure 4. Adipose Tissue Explants Isolated from ATKO Mice Display Reduced Lipolysis

(A and B) NEFA and glycerol release from explants isolated from abdominal WAT of WT or ATKO mice upon isoproterenol stimulation (10 μM, 3 hr) measured in supernatants.

(C) Immunoblot of HSL and PKA target phosphorylation and ATGL, HSL, and VCP protein expression ± iso in abdominal WAT explants.

All values are expressed as means ± SEM, n = 6–8, #p < 0.05 basal versus stimulated; *p < 0.05 WT versus ATKO. See also Figure S4.

cascade at multiple critical nodes. Of note, this function of TBLR1 seems to be largely conserved as WAT TBLR1 levels correlated with serum FFA, adrenoceptor expression in WAT and adiponectin levels also in a human patient cohort comprising both lean and obese subjects.

It is tempting to speculate that parts of TBLR1 function in WAT are mediated by a coactivating or derepressing effect on PPARγ. While PPARα or PPARβ/δ agonists failed to rescue the TBLR1 knockdown phenotype, the PPARγ agonist rosiglitazone partially restored lipolytic activity in TBLR1-deficient cells. Indeed, PPARγ has been shown to be a positive regulator of lipolytic gene expression (Rodriguez-Cuenca et al., 2012a), and the TBLR1 target gene network—at least in part—overlapped with known PPARγ targets, including HSL, aquaporin 7, and inflammatory markers (Anghel et al., 2007). This assumption is in congruence with the direct physical interaction of TBLR1 with PPARγ and its obligate heterodimerization partner RXR. Alternatively, by blocking the lipolytic response, TBLR1 deficiency could also inhibit PPARγ-mediated gene expression through an impairement in ligand availability, as activation of lipolysis by ATGL and HSL has been shown to generate essential endogenous ligands for PPAR activation (Haemmerle et al., 2011; Shen et al., 2011). Whether and how this mechanism works in parallel with the discussed TBLR1 coactivator function for PPARγ awaits further detailed studies in the future. Intriguingly, PPARγ activation by adipocyte-specific knockout of the PPARγ corepressor and TBLR1 interaction partner NCoR created an opposing phenotype to adipose TBLR1 knockout, including decreased adipose tissue inflammation and adipocyte size, increased adiponectin serum levels, and improved glucose tolerance and insulin sensitivity (Li et al., 2011). However, other nuclear-receptor/non-nuclear-receptor-dependent mechanisms distinct from PPARγ signaling are also involved in TBLR1 adipocyte function, since, in contrast to the phenotype of PPARγ$^{+/-}$ mice (Rodriguez-Cuenca et al., 2012a), we did not observe differences in adipocyte glucose uptake or lipogenesis upon TBLR1 deficiency. Indeed, we noted relative impairment of cAMP regulatory element binding protein (CREB) phosphorylation in response to TBLR1 deficiency, which might also mediate at least some aspects of TBLR1 function in WAT lipid metabolism (Qi et al., 2009).

Impaired lipolytic capacity has been considered for a long time to result in an improved overall metabolic status as reduced liberation of FFA from adipose tissue stores is commonly thought to protect peripheral tissues, including liver and skeletal muscle from FFA-dependent lipotoxicity (Frayn et al., 1996; Samocha-Bonet et al., 2012). Indeed, whole-body ATGL knockout mice display severely impaired lipolysis and subsequent accumulation of TG in WAT and other tissues, leading to overall improved insulin sensitivity and glucose tolerance (Haemmerle et al., 2006). Also, adipocyte-specific ATGL deletion leads to blunted lipolysis and severe fasting defects, but also slightly improves systemic insulin sensitivity despite mild obesity (Wu et al., 2012), and whole-body deficiency of monoglyceride lipase (MGL), which catalyzes the final step in adipose tissue TG breakdown, was found to impair lipolysis and attenuate diet-induced insulin resistance (Taschler et al., 2011).

While ATGL and MGL knockout mice parallel the TBLR1 deficient phenotype with respect to impaired lipolysis, lack of adipose tissue TBLR1 action displayed overall impairment in systemic metabolic function, supporting the notion that a decreased release of FFA from adipocytes per se—and hence elevated adipose tissue TG accumulation—does not necessarily predict an improvement in systemic insulin sensitivity as already highlighted by a number of studies in humans (Karpe et al., 2011). In contrast to the dogmatic view on FFA-mediated lipotoxicity, indeed, a series of recent studies showed that despite a more than ten-fold difference in body fat content, FFA serum levels only marginally differed between lean and obese human study groups, indicating that serum FFA levels do not fully account for metabolic complications as associated with the obese state (McQuaid et al., 2011). Instead, systemic inflammation and altered adipocytokine profile in obesity predispose for the development of insulin resistance and glucose intolerance (Hotamisligil, 2006; Rasouli and Kern, 2008).

The phenotype of the ATKO mice now points toward the possibility that indeed intrinsic deficiency in efficient FFA mobilization in WAT may be sufficient to cause systemic metabolic dysfunction, maybe as a consequence of impaired WAT metabolic flexibility upon an imbalanced equilibrium between lipid storage and mobilization (Galgani et al., 2008), and/or the simultaneous dysregulation of inflammatory and adipocytokine pathways as observed upon TBLR1 deficiency. Indeed, the TBLR1 knockout phenocopies the inhibition of lipolysis and of HSL and ATGL expression as generally observed in obese human patients (Jocken et al., 2007; Langin et al., 2005; Large et al., 1999) and strongly resembles the metabolic dysfunction in woman with polycystic ovary syndrome (PCOS), including adipocyte hypertrophy, attenuated lipolytic response to catecholamine exposure, obesity, adipose tissue inflammation, and insulin resistance (Villa and Pratley, 2011).

In line with the recently discovered function of the TBL1/TBLR1 cofactor complex in hepatic fatty acid oxidation and

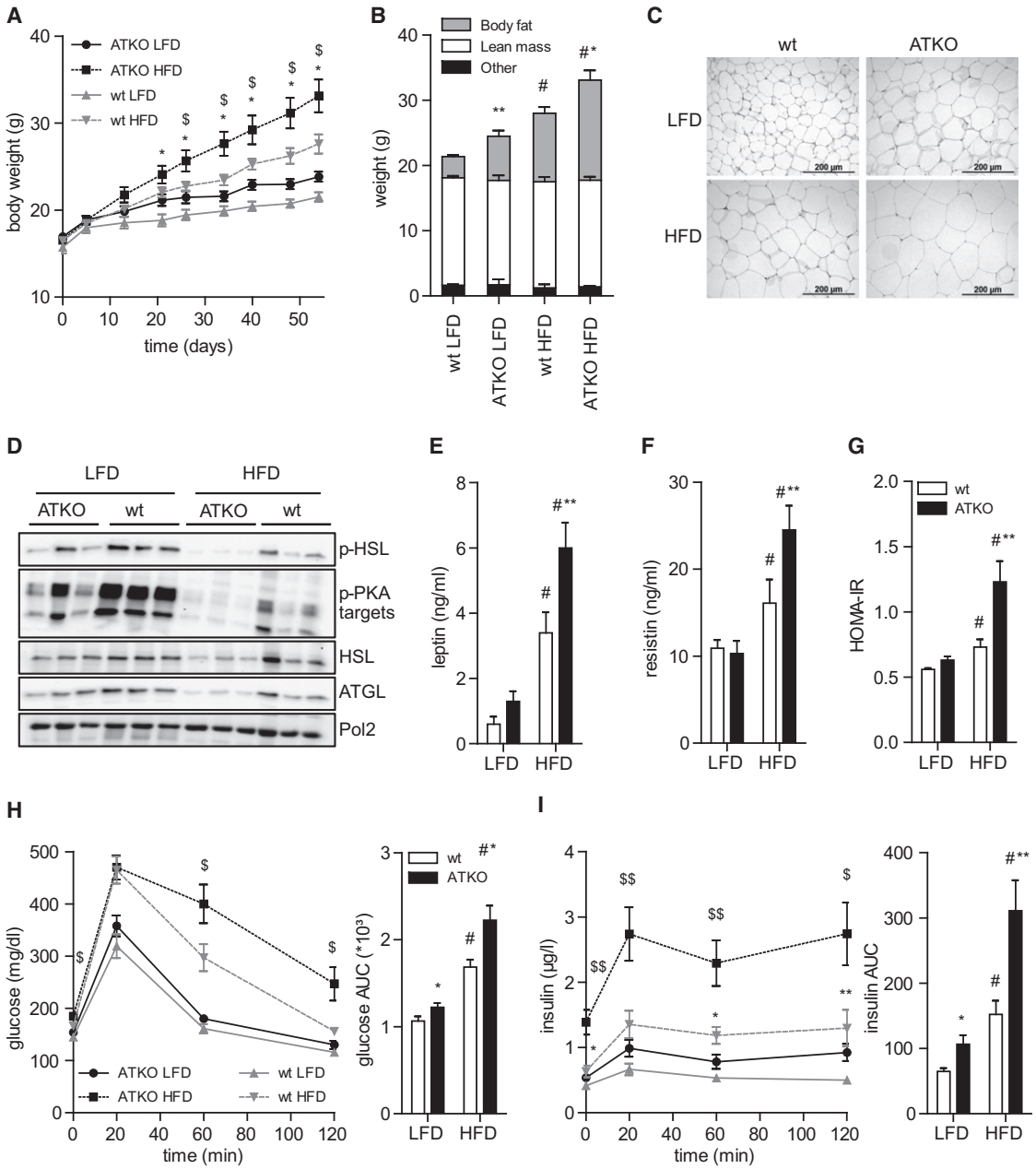

Figure 5. ATKO Mice on a High-Fat Diet Display Aggravated Adiposity and Insulin Resistance

(A) Body weight of wt or ATKO mice fed a low-fat (LFD) or high-fat (HFD) diet for 8 weeks starting from 6 weeks of age onward.

(B) ECHO-MRI body composition analysis after 8 weeks of LFD/HFD feeding.

(C) H&E-stained abdominal WAT slices.

(D) Immunoblot of p-HSL, p-PKA targets, HSL, ATGL, and RNA polymerase II in abdominal WAT.

(E and F) Serum leptin and resistin levels.

(G) Homeostatic model assessment index (HOMA-IR) of WT or ATKO mice fed a LFD/HFD for 6 weeks.

(H and I) Serum glucose and insulin levels in the same mice during an intraperitoneal glucose tolerance test and total area under the curve (AUC).

All values in bar graphs are expressed as means ± SEM, n = 8, #$p < 0.05$ LFD versus HFD; *$p < 0.05$, **$p < 0.01$ WT versus ATKO. In (A), (H), and (I), * and $ indicate significance ($p < 0.05$) within the LFD and HFD groups, respectively. See also Figure S5.

the induction of hepatic steatosis upon liver-specific TBL1/TBLR1 deficiency (Kulozik et al., 2011), our current findings overall position the TBLR1 transcriptional cofactor as an important component of the metabolic flexibility program in WAT and critical node in systemic energy homeostasis. The correlation of TBLR1 levels with metabolic parameters in human subjects indicates that tissue-specific targeting of TBLR1 may represent a conserved, yet unappreciated, approach to

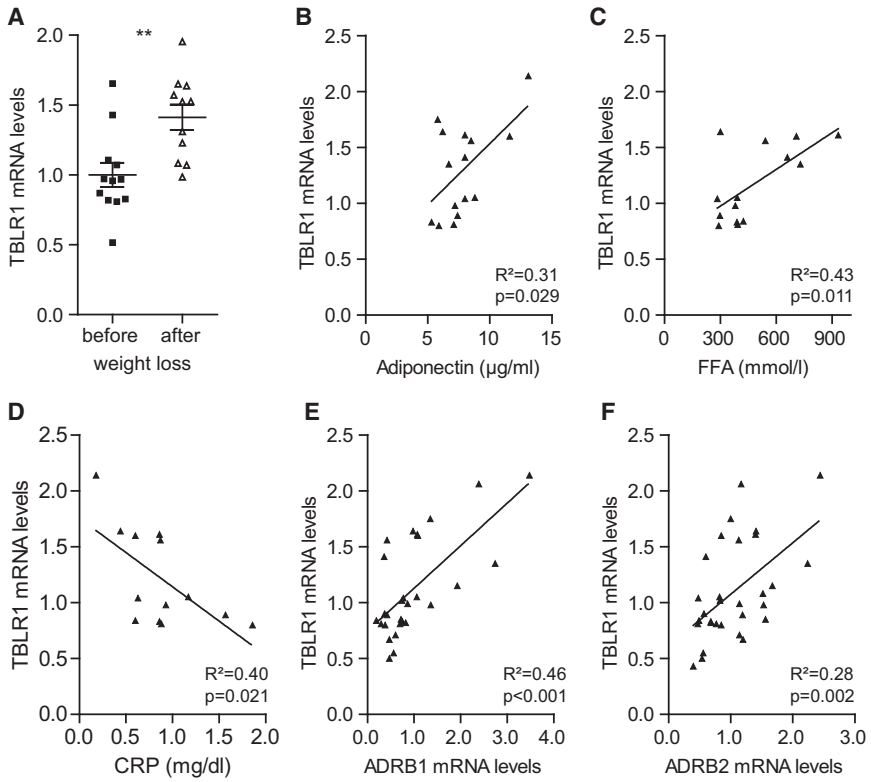

Figure 6. TBLR1 Gene Expression Is Differentially Regulated in Human WAT upon Weight Loss and Correlates with Obesity Markers and Adrenoceptor Expression

(A) TBLR1 mRNA expression in visceral WAT of female patients who underwent a weight loss program for 52 weeks (n = 12).

(B–F) Pearson correlation coefficients and p values shown for normalized WAT TBLR1 mRNA levels versus serum adiponectin levels (B), serum FFA levels (C), serum CRP levels (D), and WAT beta1- and beta2-adrenergic receptor mRNA levels (E and F) in a patient cohort representing BMIs ranging from 18.4 to 62.7 kg/m^2.

**p < 0.01. See also Figure S6 and Table S2.

counteract metabolic inflexibility in energetic stress conditions, including obesity.

EXPERIMENTAL PROCEDURES

Recombinant Adenoviruses

Adenoviruses expressing TBL1- or TBLR1-specific or nonspecific shRNA under the control of the U6 promoter or the TBLR1 complementary DNA (cDNA) sequence under control of the CMV promoter were cloned as described previously (Herzig et al., 2003) (Herzig et al., 2001). Viruses were purified by the cesium chloride gradient and dialyzed against PBS buffer containing 10% glycerol. shRNA sequences were as follows: TBL1, 5′-GCGAGGATATGGAACCTTAAT-3′; TBLR1, 5′-GGATGTCACGTCTCTAGATTG-3′; nonspecific shRNA, 5′-GATCTGATCGACACTGTAATG-3′.

Animals

Eight-week-old C57Bl6/N, NMRI, and db/db mice were obtained from Charles River Laboratories (CRL, Brussels, Belgium). Floxed TBLR1 mice were created by TaconicArtemis (Cologne, Germany) and bred with B6.Cg-Tg(Fabp4-cre)1Rev/J mice (He et al., 2003) obtained from CRL. Data obtained from female mice are shown unless stated otherwise. For starvation experiments, mice were fasted or refed for the indicated times with free access to water. For CL316,243 (CL) treatment, NMRI mice were injected intraperitoneally (i.p.) with 1 mg/kg CL316243 (Tocris Bioscience, Bristol) in 0.9% NaCl and sacrificed 3 or 8 hr later. Isoproterenol in PBS was injected i.p. into 6-month-old male mice at 10 mg/kg lean mass, and mice were sacrificed 30 min after injection. Sepsis in C57Bl6 mice was induced in 18-hr-starved animals by i.p. injection of 20 mg LPS/kg (Sigma, Munich) for 8 hr. PBS controls were starved after the injection until sacrifice to mimic the anorexia accompanying sepsis. Glucose tolerance test was performed in 6-hr-fasted animals by i.p. injection of 2 g/kg glucose. When stated HFD/LFD, mice were fed a HFD (Research Diets, New Brunswick; 60% calories from fat) or a LFD (10% calories from fat) starting at the age of 6 weeks unless stated otherwise. Indirect calorimetry was performed in PhenoMaster cages (TSE Systems, Bad Homburg, Germany) with individually housed mice at 30°C, 22°C, and 4°C, respectively. Calculations of resting metabolic rate (RMR) and body-weight-adjusted oxygen consumption were performed as described (Tschöp et al., 2012). All mice were maintained on a 12 hr light-dark cycle at 24°C with regular unrestricted diet unless stated otherwise. Organs, including liver, fat pads, and gastrocnemius muscles, were collected, snap frozen, pulverized, and used for further analysis. Total body fat content was determined by an Echo magnetic resonance imaging (ECHO-MRI) body composition analyzer (Echo Medical Systems, Houston, TX). Fecal energy content was measured with the IKA C7000 calorimeter (IKA, Staufen, Germany). Animal handling and experimentation was done in accordance with National Institutes of Health guidelines and approved by local authorities.

Histological Examinations

Tissues were fixated in 4% paraformaldehyde for 24 hr, and paraffin-embedded tissues were sliced and stained with hematoxylin and eosin (H&E) staining by standard procedures. Cell sizes were quantified in two layers of the tissue and a minimum of 100 cells/layer by Wimasis (Munich, Germany). For adipose tissue whole mount immunofluorescence, 4 mm visceral fat pad pieces were dissected and fixated in 4% paraformaldehyde for 1 hr, then blocked in 1% BSA before incubation with perilipin and F4/80 antibodies (2 μg/ml) over night at 4°C. After three washes, samples were incubated with fluorescence-conjugated secondary antibodies and imaged in a fluorescence microscope (Zeiss, Jena, Germany).

Blood Metabolites

Serum levels of glucose, insulin, glycerol, NEFAs, leptin, and resistin were determined with an automatic glucose monitor (One Touch, Lifescan, Neckargemünd, Germany) or commercial kits (Mercodia, Uppsala, Sweden; Sigma, Munich, Germany; Wako, Neuss, Germany; Merck Millipore, Schwalbach, Germany). Total NEFA levels were calculated with the assumption that a mouse has 1 ml serum.

Tissue Explants

Abdominal, inguinal and interscapular fat pads were isolated from mice and sliced into equally sized pieces of ~50 mg. Explants were washed twice in Krebs-Ringer (KR) buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, and 25 mM $NaHCO_3$) and stimulated with 10 μM isoproterenol (Calbiochem, Merck, Darmstadt) for 3 hr in KR buffer supplemented with 5% BSA, 5 mM glucose, and 25 mM HEPES/KOH (pH 7.5). NEFA and glycerol were measured from the supernatants and was calculated relative to protein content.

Cell Culture

3T3-L1 preadipocytes were cultured in low (1 g/l)-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and

1% penicillin/streptomycin (P/S) and differentiated into mature white adipocytes 2 days after confluency by the addition of 1 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone, and 1/1000 volume ABP (50 mg/ml L-ascorbate, 1 mM biotin, 17 mM pantothenate) in high (4.5 g/l)-glucose DMEM containing 10% FCS and 1% P/S. Primary stromal-vascular fractions (SVFs) were isolated from mouse inguinal fat depots, cultured, and differentiated into mature primary adipocytes as described (Rodeheffer et al., 2008). Cells were transduced with recombinant adenoviruses at a multiplicity of infection (MOI) of 1,000 (primary adipocytes) or 500 (3T3-L1 adipocytes) with 0.5 μg/ml poly-L-lysine. For analysis of adipocyte differentiation upon TBL/R1 knockdown, confluent 3T3-L1 preadipocytes were transduced with adenoviruses carrying respective shRNAs at an MOI of 500 2 days prior to induction of differentiation. Triglyceride accumulation was measured by oil red O staining. In brief, cells were fixated in 10% formaldehyde in PBS for 1 hr, washed in 60% isopropanol, and dried. Triglycerides were stained with six parts oil red O solution (0.7 g oil red O in 200 ml isopropanol) and 4 parts H_2O. For luciferase assays, 3T3-L1 preadipocytes were transfected with plasmids encoding TBLR1 shRNA or overexpression constructs, PPRE, HSL, and ATGL promoter sequences, PPAR and RXR expression constructs, and β-galactosidase cDNA with Lipofectamine (Invitrogen). Mature adipocytes were transfected with a NEON transfection device (Invitrogen). Primer sequences were as follows: pATGLfor, 5′- GACGGC TAGCTAGGACCCCGGCTCTCTTTTCAG-3′; pATGLrev, 5′- GCGAAAGCTTC GGCGGAGGCGGAGACGCTGTGC-3′; pHSLfor, 5′- CCCCACGCGTGTCAT TATGATCAGCCCA-3′; pHSLrev, 5′- TTTTAGATCTCAAGCTGGCACAGCA GGTCTG-3′. Lipolysis assays were performed in mature adipocytes 9 to 12 days after induction of differentiation. After preincubation in KR buffer for 2 hr, cells were stimulated with 10 μM isoproterenol (iso), 10 μM norepinephrine (NE), 100 nM procaterol (pro), 10 μM CL, 10 μM forskolin (fsk), or 0.5 mM IBMX for 3 hr in KR buffer supplemented with 5% BSA, 5 mM glucose, and 25 mM HEPES/KOH (pH 7.5). cAMP assays were performed with standard kits (Cayman Chemicals, Ann Arbor). Glucose uptake and lipogenesis were stimulated by 10 nM insulin in KR buffer supplemented with 3% BSA, 25 mM HEPES/KOH (pH 7.5) and 1 mM glucose spiked with 0.1 μCi D-[^{14}C(U)]-Glucose (Perkin Elmer, Waltham). Cells were lysed with 0.5 M NaOH, and defined amounts were used to measure overall glucose uptake or to isolate TG and measure lipogenesis by counting of ^{14}C lipid incorporation. Glucose uptake and lipogenesis were calculated as the percent induction by insulin. Palmitate oxidation was measured from adipocytes treated with 200 μM palmitate spiked with 0.5 μCi/ml ^{3}H palmitate in KR buffer supplemented with 3% BSA, 5 mM glucose, and 25 mM HEPES/KOH (pH 7.5) for 3 hr. The aqueous phase of the supernatants was isolated by addition of 2:1 chloroform:methanol, and disintegrations per minute were counted in a scintillation counter. For protein and TG measurements, cells were lysed as described (Haas et al., 2009). NEFAs, TG, and glycerol were measured from cell lysates or supernatants with commercial kits (Wako, Neuss; Sigma, Munich). Proteins were quantified by the BCA method (Fisher Scientific, Schwerte). TG, NEFAs, and glycerol were normalized to protein levels.

Protein Analysis

Proteins were extracted from frozen organ samples or cultured adipocytes as described (Rose et al., 2011), and extracts were separated on 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Western blot assays were performed with antibodies specific for Actin, Pol II, RXR, ADRB2, ADRB3 (Santa Cruz, Heidelberg), TBLR1 (Novus Biologicals, Littleton), Perilipin A, TBL1, VCP, PPARγ (Abcam, Cambridge), GFP (Invitrogen, Karlsruhe), AKT, p-AKT Ser473, p-HSL Ser660, ATGL, p-PKA substrate (Cell Signaling, Danvers), or HSL (Chemicon, Hofheim). The ratio of p-HSL/HSL was calculated with ImageJ. For immunoprecipitation, differentiated 3T3-L1 adipocytes on 10 cm plates were lysed in 1 ml lysis buffer (20 mM HEPES/KOH [pH 7.4], 125 mM NaCl, 0.5 mM EDTA, 0.1% Igepal, and 10% Glycerol) containing protease inhibitor for 30 min on ice. After spinning (13,000 rpm, 4°C, 30 min), supernatants were collected and incubated with protein A/G agarose beads and 4 μg of the respective antibody over night at 4°C on a rotating wheel. Protein-bound beads were washed three times with lysis buffer, and proteins were eluted by the addition of SDS sample buffer and subjected to immunoblotting.

Quantitative Taqman RT-PCR

Total RNA was extracted from frozen organ samples or cultured adipocytes with QIAzol and the RNeasy (QIAGEN, Hilden) kit. RNA was extracted from human adipose tissue by Trizol. cDNA was prepared by reverse transcription with M-MuLV enzyme and Oligo dT primer (Fermentas, St. Leon-Rot). cDNAs were amplified with assay-on-demand kits and an ABI StepOnePlus sequence detector (Applied Biosystems, Darmstadt). TBLR1 exon 5 was detected by SYBR PCR using the following primers: TBLREx5for, 5′-TGCTGCC GCCACTAACCAGC-3′; TBLREx5rev, 5′-TGTGCTCCATTCTCCTCCCC-3′. RNA expression data were quantified according to the delta Ct method as described (Livak and Schmittgen, 2001) and normalized to levels of TATA-box binding protein RNA for mouse or ubiquitin C (UBC) for human adipose tissue.

Microarray Analysis

Gene expression profiling was performed in 3T3-L1 adipocytes transduced with TBLR1 or control shRNA carrying adenoviruses. RNA isolation, cRNA synthesis, and hybridization to Mouse Genome 430 2.0 arrays (Affymetrix, Freiburg) were performed according to the manufacturer's recommendations. Three arrays per group were hybridized. CustomCDF by Brainarray with Entrez based gene definitions (Entrez basic version 13) was used to annotate the arrays (Sandberg and Larsson, 2007). The Raw fluorescence intensity values were normalized by application of quantile normalization. Differential gene expression was analyzed based on ANOVA with a commercial software package (SAS JMP8 Genomics, version 4, SAS Institute, Cary, NC). A false positive rate of 0.05 with FDR correction was taken as the level of significance. Pathways belonging to various cell functions were obtained from public external databases (KEGG, http://www.genome.jp/kegg/). A Fisher's exact test was performed to detect the significantly regulated pathways.

Human Subjects

For the fasting study, 12 obese subjects underwent a weight reduction program (OPTIFAST 52[9]) for 52 weeks (BMI = 45.6 ± 2.5 kg/m^2 before, 39.0 ± 2.2 kg/m^2 after) and WAT TBLR1 mRNA expression was measured before and after the weight reduction; mRNA levels were normalized to 36B4. This study has been approved by the ethics committee at Heidelberg University, and all patients gave written informed consent. For the second study, TBLR1 mRNA expression in omental WAT of 31 female patients (BMI ranging from 18.4 to 62.7 kg/m^2) was normalized to UBC mRNA levels and correlated to serum parameters or WAT adrenoceptor expression with Pearson correlation. This study has been approved by the ethics committee of the Medical University of Vienna and the General Hospital Vienna, and all patients gave written informed consent.

Statistical Analysis

Statistical analyses were performed with a two-way ANOVA with Bonferroni-adjusted posttests or Student's t test in one-factorial designs, respectively. $p < 0.05$ was considered statistically significant. Error bars in figures represent the SEM.

ACCESSION NUMBERS

Microarray data sets have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE43658.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.02.010.

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